Diurnal variation of anterior scleral and conjunctival thickness

Running Head: Anterior scleral diurnal variations

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Date of Submission: 25th January 2016

Word Count: 4427
Number of Tables: 2
Number of Figures: 6
Keywords: Sclera, Conjunctiva, Diurnal, Anterior segment, Optical coherence tomography

Disclosure: The authors report no conflict of interest and have no proprietary interest in any of the material presented in this article.

Acknowledgements: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Abstract

Purpose: To examine whether anterior scleral and conjunctival thickness undergoes significant diurnal variation over a 24-hour period.

Methods: Nineteen healthy young adults (mean age 22 ± 2 years) with minimal refractive error (mean spherical equivalent refraction -0.08 ± 0.39 D), had measures of anterior scleral and conjunctival thickness collected using anterior segment optical coherence tomography (AS-OCT) at seven measurement sessions over a 24-hour period. The thickness of the temporal anterior sclera and conjunctiva were determined at 6 locations (each separated by 0.5 mm) at varying distances from the scleral spur for each subject at each measurement session.

Results: Both the anterior sclera and conjunctiva were found to undergo significant diurnal variations in thickness over a 24-hour period (both $p < 0.01$). The sclera and conjunctiva exhibited a similar pattern of diurnal change, with a small magnitude thinning observed close to midday, and a larger magnitude thickening observed in the early morning immediately after waking. The amplitude of diurnal thickness change was larger in the conjunctiva (mean amplitude 69 ± 29 μm) compared to the sclera (21 ± 8 μm). The conjunctiva exhibited its smallest magnitude of change at the scleral spur location (mean amplitude 56 ± 17 μm) whereas the sclera exhibited its largest magnitude of change at this location (52 ± 21 μm).

Conclusions: This study provides the first evidence of diurnal variations occurring in the thickness of the anterior sclera and conjunctiva. Studies requiring precise measures of these anatomical layers should therefore take time of day into consideration. The majority of the observed changes occurred in the early morning immediately after waking and were of larger magnitude in the conjunctiva compared to the sclera. Thickness changes at other times of the day were of smaller magnitude and generally not statistically significant.

Introduction

The development and advancement of optical coherence tomography (OCT) imaging technology, now allows precise, high resolution *in-vivo* measures of many of the eye's anatomical structures.[1] Although OCT imaging has primarily been used in research and clinical practice for imaging posterior structures such as the retina and choroid,[2] OCT is being used increasingly more in the assessment of anterior segment structures such as the cornea[3,4] and anterior chamber.[3] Anterior segment OCT (AS-OCT) also allows the imaging and measurement of the anterior sclera and conjunctiva, providing high resolution, non-invasive *in-vivo* measures of these structures.[5,6]

Prior to the advent of AS-OCT, methods to assess anterior scleral thickness either had relatively low resolution (e.g. magnetic resonance imaging,[7] scleral profile photography[8]) and/or involved varying degrees of invasiveness in order to provide measurements (ranging from methods requiring topical anaesthesia and contact with the eye such as ultrasound,[9] to methods only possible on sectioned scleral tissue such as light microscopy[10]). Much of our current knowledge of the dimensions of the human sclera are therefore based upon *ex-vivo* measures of the sclera,[7-10] which may not provide an exact representation of the *in-vivo* dimensions of the sclera (due to potential changes occurring in the scleral structure post-mortem and/or related to the tissue fixation and sectioning process). Furthermore, such measurements do not provide an assessment of the natural dynamics occurring in these tissues.

A number of recent studies have used AS-OCT to examine the *in-vivo* changes in anterior scleral morphology associated with the presence of glaucoma,[11] glaucoma filtration surgery,[12] ocular therapeutic procedures,[13,14] keratoconus,[15] contact lens wear[16] and the presence of inflammatory conditions such as scleritis and episcleritis.[17] A small number of studies have also reported upon the thickness characteristics of the anterior sclera in normal adult eyes,[6,18] and others have examined the normal dimensions of the bulbar conjunctiva using AS-OCT.[5,19]

Although these recent studies have expanded knowledge of the normal *in-vivo* dimensions of the anterior sclera and conjunctiva, and the factors that may influence

these structures, the natural changes occurring in anterior scleral and conjunctival thickness over the course of the day have not been previously investigated. Understanding the natural diurnal variations occurring in these tissues is of fundamental importance for clinical and research applications aiming to precisely assess conjunctival and scleral dimensions, and for the reliable interpretation of measurements, particularly when changes in scleral and conjunctival thickness are evaluated longitudinally over time. In this study we used AS-OCT in order to examine the diurnal changes in scleral and conjunctival thickness over a 24-hour period in a group of young, healthy adult participants with minimal refractive error.

Methods

Subjects and Procedures

In this study, AS-OCT was used to assess the diurnal variations occurring in the thickness of the anterior sclera and conjunctiva of nineteen healthy young adult subjects (mean (SD) age: 21.5 (2.3) years). Ten of the 19 subjects were female. A sample size of 19 was employed, since it is consistent with previous studies documenting statistically significant diurnal variations in other anterior segment parameters,[20-23] that have utilised sample sizes ranging from 9 to 20 participants. Approval from the Queensland University of Technology Human Research Ethics Committee was obtained prior to commencing the study and all subjects were treated in accordance with the tenets of the declaration of Helsinki and provided written informed consent to participate.

All subjects initially underwent an ophthalmic examination to determine their refractive status and ensure they had normal vision and ocular health. The non-cycloplegic subjective spherical equivalent refractive error (SER) of each subject's right eye was close to emmetropia (between -0.75 DS and +1.00 DS), with a best corrected visual acuity of 0.00 logMAR or better in both eyes. The mean (SD) SER was -0.08 (0.39) D, and the mean astigmatic refractive error was -0.32 (0.39) DC. No subject exhibited amblyopia, strabismus or anisometropia >1.00D, and none were contact lens wearers. All subjects reported to be in good general health, with no history of any systemic disease or use of systemic medications known to effect refractive status.

Each of the 19 eligible participants then had images of their right eye's temporal anterior sclera and conjunctiva taken with AS-OCT at 7 different measurement sessions conducted over a 24-hour period (across 2 days). Only the temporal anterior segment was imaged to reduce the potential impact of the presence of sun-related scleral and conjunctival changes (e.g. pterygia, pingueculae) upon the study, since these changes most commonly involve the nasal sclera and conjunctiva.[24,25] The measurement sessions occurred at the following mean (SD) times (hrs:min): 08:56 (00:15), 12:26 (00:09), 16:13 (00:26), 19:28 (00:12), 22:58 (00:08), 05:33 (00:08), 08:43 (00:14). The first measurement session was conducted at least 2 hours after the subjects' reported time of waking on that day, and each subsequent measurement session occurred approximately every 3.5 hours throughout the first measurement day. After the last night-time measurement on that day (22:58 (00:08)), subjects then slept in individual darkened rooms within the research laboratory. The next morning (05:33 (00:08)), approximately 6.5 hours later, each subject was woken for the next measurement. To reduce the influence of postural changes on this measurement, subjects sat upright for 5 minutes with their eyes closed before measurements were collected.[26] The final measurement (08:43 (00:14)) then occurred approximately 3.5 hours later on the morning of the second measurement day (i.e. 24 hours after the first measurement session).

All anterior segment images were collected using the Heidelberg Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) instrument with anterior segment module. This instrument is a spectral domain OCT device that utilizes a super luminescent diode with central wavelength of 870nm for OCT imaging, and provides cross-sectional images of the anterior eye with an axial resolution of 3.9 µm, transverse resolution of 14 µm and a scanning speed of 40,000 A-scans per second. In addition to the OCT images, this instrument also uses a confocal scanning laser ophthalmoscope (SLO) to provide *en-face* images of the anterior eye. This allows automatic real-time eye tracking during OCT image acquisition (to facilitate frame averaging in order to improve image quality and reduce signal to noise ratio). The SLO image was also used to register the OCT scans using features identified in the *en-face* image.

At each measurement session, two volumetric scans were collected using the instrument's scleral imaging mode (which utilises enhanced depth imaging to optimise the image of the sclera) and high resolution (HR) scanning (with each cross sectional image consisting of 1024 by 678 pixels, equating to an 11.1 mm scan length and 2.6 mm scan depth). The dimensions of the volume scan encompassed an 11.1 by 2.8 mm (20° by 5°) region of the anterior eye and consisted of 11 horizontal cross-sectional OCT images (each cross-sectional image was the average of 25 B-scans) each separated vertically by 277 microns (Figure 1). The OCT images produced by this instrument are scaled using the device's proprietary algorithms, to allow geometric measures of tissue thickness, assuming a refractive index of 1.40. Only images with a quality index (QI) of >25dB were included (mean QI of all images from all sessions was 38 (4) dB). In order to image the temporal anterior sclera, subjects fixated upon an external fixation target (a green light emitting diode) (located 30° nasally at a distance of 4 metres from the subject's eye) during imaging.

Data Analysis

Following data collection, the OCT images were analysed using custom written software to determine the anterior scleral and conjunctival thickness at each measurement session. The OCT imaging and analysis procedures performed in the study are summarised in Figure 1. Initially, the central cross-sectional image within the volumetric scan collected at the first measurement session exhibiting the best image quality was selected. Two experienced, independent masked observers then performed analysis on the *en-face* SLO images associated with the volumetric scans collected at each measurement session, to determine the scan position at each measurement session that corresponded to the central scan from the first measurement session. Each observer selected 4 anatomical landmarks (typically blood vessel bifurcations or crossings) in the first measurement session *en-face* image, and the location of these same landmarks within each of the subsequent collected images. The location of these common points allowed the magnitude of translation and rotation required to align each subsequent image with the first measurement session image to be calculated. This information was used to determine which B-scan in each of the subsequent measurements corresponded closest to the location of the central B-scan of the first measurement session image.

The image at each session displaying the smallest magnitude of rotation with respect to the session 1 image was selected for further analysis. In cases where there was negligible difference in rotation between the 2 images at a measurement session, the best quality image of the two measurements was selected for analysis. The *en-face* image analysis from these two observers exhibited good reliability for the between image rotation (mean (SD) inter-observer difference in rotation estimate of 0.08 (0.27)°). For the final images included in the analysis, the mean absolute rotation between images was 0.98 (0.85)°.

Following the initial analysis to register the best quality central cross-sectional image from the first measurement session, with the corresponding cross-sectional images at each subsequent measurement session, the OCT images included for the final analysis were segmented using a semi-automated procedure. Initially, an automated algorithm based upon graph theory was used to delineate the anterior conjunctival boundary and posterior scleral boundary across the entire width of the image. Two experienced, independent masked observers then further analysed each image to correct any automated segmentation errors, and to also manually segment the anterior scleral boundary (the junction between the sclera and the episclera defined by the posterior boundary of the hypo-reflective episcleral vascular plexus) and locate the position of the scleral spur (SS) in each image using the ciliary muscle method described by Seager et al.[27] The segmentation information from the analysis of each observer on each image was averaged, and then used to derive a profile of the conjunctival thickness (defined as the axial distance from the anterior conjunctival boundary to the anterior scleral boundary) and the scleral thickness (the axial distance from the anterior scleral boundary to the posterior scleral boundary) with respect to the SS location, for each of the seven measurement sessions. This thickness profile was then used to derive conjunctival and scleral thickness measurements at 5 discrete locations situated at 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 mm from the SS.

A repeated measures analysis of variance (ANOVA) with 2 within-subject factors (measurement time and measurement location with respect to the SS) was then carried out to examine whether the temporal anterior conjunctival thickness and scleral thickness varied diurnally or with location away from the SS. Degrees of

freedom were adjusted using the Greenhouse-Geisser correction to account for violations of the sphericity assumption. Pairwise comparisons with Bonferroni correction were carried out to explore any significant main effects and interactions in the ANOVA. P-values <0.05 were considered statistically significant. The reliability and repeatability of the thickness measures were assessed through analysis of the scleral and conjunctival thickness measures from the two observers. Additionally, the within-session repeatability of the measures was assessed by deriving the scleral and conjunctival thickness from the two repeated OCT scans collected at the first measurement session. Bland-Altman[28] analysis was used to examine both the inter-observer measurement repeatability and the within-session repeatability. The within-subject standard deviation was also calculated[28] and expressed as the coefficient of variation (COV, the within-subject SD divided by the mean and expressed as a percentage) as an additional metric of repeatability, for the within-session and inter-observer data.

Results

Measurement repeatability

Table 1 and Figure 2 illustrate the within-session and inter-observer repeatability of the measures of scleral and conjunctival thickness. These analyses revealed good inter-observer and within-session repeatability, with the COV being 3% and 5% for the scleral and conjunctival thickness respectively. However, the width of the 95% limits of agreement of the difference between repeated measures within a session, and between observers (Figure 2), suggests a moderate degree of variability associated with the measurement procedures for some individual thickness measures.

Average conjunctival and scleral thickness profile

The thickness profile of the anterior conjunctiva ($F_{1.7, 30.4} = 10.7$, $p<0.001$) and sclera ($F_{1.3, 22.7} = 120.2$, $p<0.001$) both exhibited significant variation with distance from the SS (Table 2). Figure 3 illustrates the average thickness profile of the anterior temporal conjunctiva and sclera for all 19 subjects (averaged across all 7 measurements sessions). Across the 2.5 mm measurement zone, the conjunctiva exhibited its minimum thickness at the SS (mean (SD) thickness 226 (31) µm), and

maximum thickness 1 mm away from the SS (262 (39) µm) (Figure 3a). Pairwise comparisons revealed that the conjunctival thickness at the SS location was significantly thinner than the 0.5 mm and 1.0 mm location thickness measures. The 1.0 mm location thickness was significantly greater than the SS location and 2.5 mm location. Over the 2.5 mm measurement zone, the sclera was found to be thickest at the SS (668 (40) µm), and reached a minimum in thickness at 1.5 mm from the SS (466 (52) µm) (Figure 3b). Pairwise comparisons revealed that the scleral thickness at the SS location was significantly greater than all other locations, and that the 1.5 mm location thickness was significantly thinner than the SS, 0.5, 1.0 and 2.5 mm location scleral thickness measures.

Diurnal variations in conjunctival and scleral thickness

Significant diurnal variations were observed in both conjunctival ($F_{3.0, 53.6} = 20.7$, $p<0.001$) and scleral thickness ($F_{3.9, 69.9} = 4.7$, $p = 0.002$). Figure 4 illustrates the mean changes in conjunctival and scleral thickness (averaged across all measurement locations) from baseline over the 24-hour study period, and demonstrates a similar pattern of diurnal thickness change in both the sclera and conjunctiva. On average, a minimum in thickness was observed in both tissues at the second measurement session (day 1, mean time 12:26), and a maximum in thickness at the sixth measurement session (day 2, mean time 05:33) immediately after waking. For the sclera, statistically significant differences were observed between the thickness at the sixth measurement session (day 2, mean time 05:33 am) and the second measurement session (day 1, mean time 12:26 pm), whereas for the conjunctiva, the thickness at the sixth measurement session was significantly greater than all other sessions, and the thickness at the fifth measurement session (day 1, mean time 22:58 pm) was significantly greater than the first (day 1, mean time 08:56 am) and second measurement session (day 1, mean time 12:26 pm) (all $p<0.05$). For both the sclera and the conjunctiva, the thickness at the first measurement session was not significantly different to the thickness measured approximately 24 hours later at the final measurement session.

The magnitude of the observed daily changes were approximately 2.3 times greater in the conjunctiva (mean amplitude of change 69 (29) µm) compared to the sclera (21 (8) µm). The difference between these mean amplitudes of change (calculated

from each individual subject's diurnal amplitude) and the amplitude of thickness change observed in the group mean thickness change plots of Figure 4 is indicative of some between subject variations in the timing of the maximum and minimum thickness. For the scleral thickness, 79% of subjects exhibited a thickening with respect to the baseline measurement at the early morning measurement session (session 6), with 58% of subjects exhibiting their maximum thickening at this session. For the conjunctival thickness, 100% of subjects exhibited an increase in thickness at measurement session 6, with 68% of subjects exhibiting their maximum thickness at this session. Figure 5 (and supplemental file) illustrates the variations observed in the OCT images and tissue thickness at each session for an individual representative subject.

The mean diurnal changes at each of the 6 measurement locations with respect to the SS for both the conjunctival and scleral measures are shown in Figure 6. A similar pattern of change was found in each of the different measurement locations, with all locations typically exhibiting a small magnitude thinning at the second measurement session and a larger magnitude thickening at the sixth measurement session for both the conjunctiva and sclera. For the conjunctival thickness data, a significant measurement location by time interaction was observed ($F_{5.9, 106.4} = 4.5$, $p<0.001$), which was driven by the larger magnitude diurnal changes observed in the outer locations (1.0 to 2.5 mm locations) compared to the SS location. For the scleral data, the opposite trend was observed, with a greater magnitude of diurnal change seen at the SS location compared to the outer locations, but the time by measurement location interaction did not reach significance ($F_{9.0, 162.6} = 0.8$, $p=0.59$).

To further examine these changes, the diurnal amplitude of change was also calculated (the difference between the maximum and minimum change in thickness over the 24-hour period) for each subject at each measurement location (Table 2). Repeated measures ANOVA revealed significant variations in the diurnal amplitude of change associated with measurement location, for both the conjunctival thickness ($F_{2.3, 42.1} = 16.5$, $p<0.001$) and scleral thickness ($F_{2.9, 53.0} = 12.2$, $p<0.001$). For the conjunctival thickness, the largest magnitude diurnal amplitudes were observed at the 1.5 and 2.0 mm locations, and pairwise comparisons revealed that these changes were significantly greater ($p<0.05$) than those observed at the SS and 0.5

mm locations. On the other hand, the sclera exhibited its greatest magnitude of diurnal thickness change at the SS location, and these changes were significantly greater than those observed at the 1.0, 1.5, 2.0 and 2.5 mm locations (all $p<0.05$).

Discussion

This study provides the first evidence that *in-vivo* measurements of anterior conjunctival and scleral thickness demonstrate significant variations over a 24-hour period. The anterior sclera and conjunctiva exhibited a similar pattern of change over the course of the day, with a small magnitude thinning of both tissues observed close to mid-day, and a larger magnitude thickening observed in the early morning, immediately after waking. These findings suggest that future studies requiring highly precise measures of anterior scleral and/or conjunctival thickness, should take the time of day into account. However, it should be noted that statistically significant changes in thickness were only observed in the early morning immediately after waking for the sclera, and were seen at the early morning measurement and the late night measurement for the conjunctiva, with thickness changes at other times of the day being of smaller magnitude and within the repeatability of the measurement technique. This suggests that many clinical and research applications requiring anterior scleral and conjunctival measures are unlikely to be substantially affected by these changes, if measurements are collected within normal office hours.

The increases in anterior scleral and conjunctival thickness observed immediately after waking are most likely related to changes in the ocular environment associated with prolonged overnight eye closure. It is well established that eyelid closure results in a reduction of oxygen available to the ocular surface,[29] and this closed eye hypoxia is thought to be one of the major factors associated with the well documented swelling that occurs in both the central[20,21] and peripheral cornea[23] immediately after waking. Duench et al[22] have also reported upon significant increases in bulbar conjunctival blood flow (and associated increased redness and temperature of the conjunctiva) immediately after waking, and suggested that reduced oxygen at the ocular surface due to overnight lid closure may stimulate an increase in conjunctival blood flow in order to deliver more oxygen to the ocular surface. It is therefore conceivable that a hypoxia induced increase in blood flow to

the anterior eye may also underlie the increases in tissue thickness that we observed upon waking in our current study.

A range of changes are also documented to occur in the tear-film with overnight eye closure.[30-34] Tear film osmolarity is reported to be at a minimum immediately after waking,[30,31] and there is also evidence to suggest an increase in tear film inflammatory markers/mediators occurs with overnight eyelid closure,[32,33] consistent with a degree of sub-clinical inflammation in the closed eye environment.[34] These findings support the possibility that changes in tear film osmolarity and/or inflammatory processes may also have contributed to the swelling we observed in the anterior sclera and conjunctiva immediately after waking.

Although the sclera and conjunctiva exhibited a similar pattern of diurnal change in thickness, the magnitude of change in the conjunctiva was substantially greater than that seen in the sclera. This is most likely due to the different compositions of the two tissues, since the highly vascular nature of the anterior conjunctiva would be expected to result in greater dynamic variations in thickness compared to the relatively avascular sclera. Interestingly, we also observed regional differences in the diurnal changes in thickness of the sclera and conjunctiva. The conjunctiva exhibited its smallest amplitude of diurnal change at the SS location, and the sclera its largest amplitude of change at the same location. The conjunctiva's overall thickness was also at a minimum at the SS, which may render this region less prone to larger diurnal variations compared to thicker regions containing greater amounts of vascular tissue. The sclera at the SS location, is continuous with the peripheral corneal stroma, as well as with the anterior chamber, which may promote a greater osmotic flow of fluid into the sclera in this region, and may have contributed to the sclera's larger magnitude thickness variations at the SS location.

A small number of recent studies have reported upon anterior scleral thickness using AS-OCT in normal adults with a variety of ages.[6,15,18] Ebneter et al[6] and Schlatter et al[15] examined scleral thickness at 2.0 mm from the SS, in supero-temporal, supero-nasal, infero-temporal and infero-nasal quadrants and reported that scleral thickness varied significantly between quadrants, being thickest in superior quadrants, and thinner in inferior quadrants. Although our current study measured at a single scleral

location (i.e. a temporal location as opposed to four quadrants) our average temporal scleral thickness at 2.0 mm from the SS (476 µm) agrees closely with the average of the two temporal quadrants examined in the studies of Ebneter et al[6] (469 µm) and Schlatter et al[15] (458 µm) in their populations of normal adults. Of note, Pekel et al[18] also examined the temporal scleral thickness in normal adults, and reported substantially higher thickness values compared to our results and to those of Ebneter et al[6] and Schlatter et al.[15] This difference is likely due to the measurement locations (which were defined with respect to the limbus by Pekel et al,[18] rather than the SS) and definitions of scleral thickness (which appears to extend from the posterior sclera through to the posterior surface of the conjunctival epithelium), which differed to our current study and to the measurements of Ebneter et al[6] and Schlatter et al.[15] Zhang et al[19] have also recently reported upon bulbar conjunctival thickness in normal adults with AS-OCT (in an infero-temporal location ~3 mm from the limbus), and their reported average thickness of 258 µm in 21-30 year olds, is comparable to our temporal conjunctival thickness at 2.5 mm from the SS of 241 µm.

In the current study, to allow more ready comparisons with previous work investigating anterior scleral thickness with this OCT instrument, analysis to determine geometric thickness values was performed upon OCT images that were scaled by the device's proprietary algorithms. This approach assumes the scleral and conjunctival tissue has a common refractive index (n = 1.40). Although this assumption is consistent with a number of previous studies of anterior scleral thickness using a range of different devices,[6,11,13-16,18] there is evidence from in-vitro studies that the bulbar conjunctiva and sclera exhibit some differences in their optical properties.[35] However, we estimate that accounting for the small difference in refractive index between the sclera (n = 1.41) and conjunctiva (n = 1.38) in the determination of thickness values would result in only a -0.7% and +1.5% change in the scleral and conjunctival thickness estimates respectively. This equates to less than 1 µm change in the estimated diurnal thickness changes reported in our current analysis. OCT images may also be affected by geometric and refractive distortions,[36] which could also potentially influence the thickness values in this study. However the effects of these distortions on the thickness values would be expected to be relatively constant between the measurement sessions, which suggests their effect on the changes in thickness observed would be minimal. It should also be

noted that the relatively flat curvature of the anterior sclera, and small refractive index difference between the sclera and conjunctiva (0.03) would predict only small effects of refractive distortion upon axial thickness measures, which is the likely reason why the majority of previous studies of anterior scleral,[6,11,13-16,18] conjunctival[19] and ciliary body thickness[37,38] in the anterior segment do not appear to have specifically adjusted for this factor in their measurements.

A limitation of our current study is that our measurements were made in only the temporal region of the anterior eye. Given the documented regional variations in anterior scleral thickness,[6,15,16] it may be of interest in future research to examine whether different locations exhibit differences in the diurnal changes. Further research is also required, to more comprehensively understand how rapidly the scleral and conjunctival thickness returns to baseline levels after waking, since the measurement sessions in the current study were separated by ~3.5 hours. Our protocol also only included young healthy adults with minimal refractive error. Therefore, further research is required to understand whether the pattern of diurnal variations in anterior sclera and conjunctiva differs with age, refractive error and/or the presence of ocular disease. The sclera is well documented to undergo changes in its structural, biochemical and biomechanical properties associated with the development and progression of myopia.[39,40] Examining whether diurnal variations in scleral thickness are influenced by the presence, development and progression of myopia, particularly in young subjects whose eyes are undergoing more rapid growth will therefore be an interesting area of future research. Previous studies indicate that many of the structural scleral changes associated with myopia primarily affect the posterior sclera,[7,10] therefore developments in imaging technology to allow more posterior scleral regions to be reliably imaged and measured may be required to comprehensively understand the influence of myopia upon diurnal scleral changes.

In conclusion, this study demonstrates that the thickness of the anterior sclera and conjunctiva undergo significant changes over a 24-hour period. Studies requiring precise measures of these structures should therefore take time of day into consideration. These changes were of larger magnitude in the conjunctiva compared to the sclera, and were most prominent immediately after waking. The

changes observed during the day were of smaller magnitude and within the repeatability of the measurement technique.

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Figures:

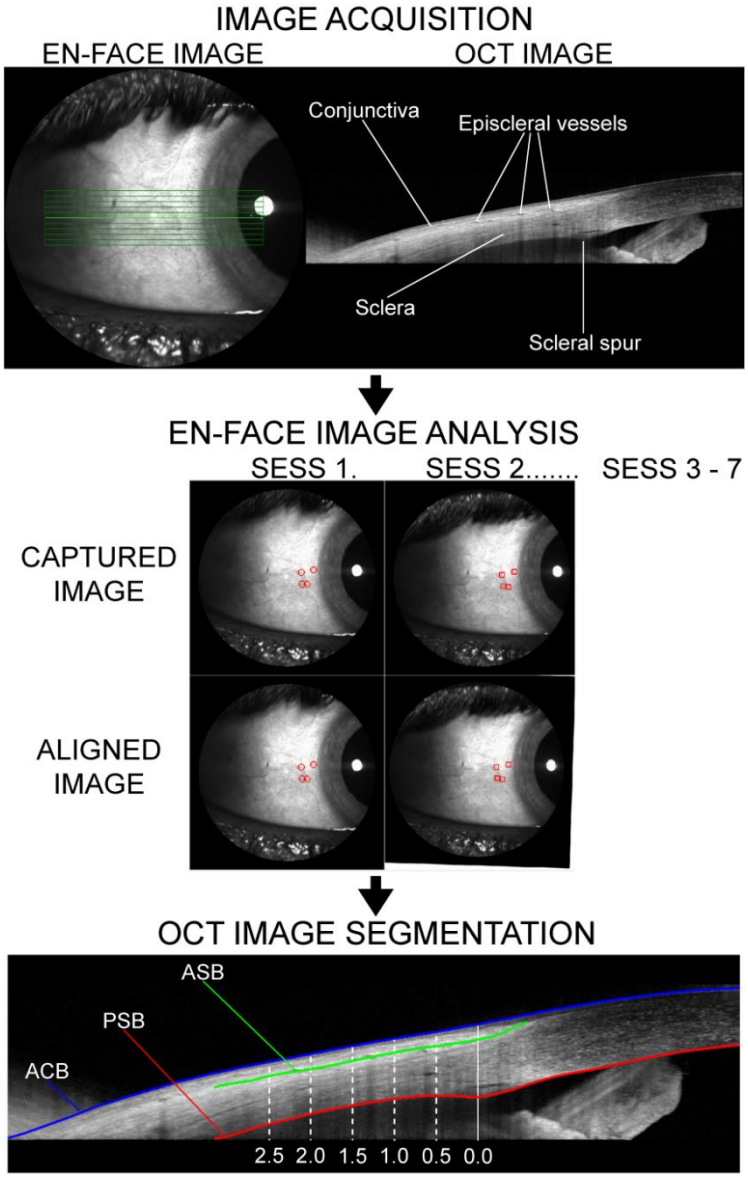

Figure 1: Overview of the OCT image acquisition and analysis procedures performed to determine conjunctival and scleral thickness. At each session 2 x volumetric OCT scans were collected from the temporal limbus of the right eye (covering an 11.1 by 2.8 mm region, with 11 cross-sectional OCT images) (top). The *en-face* images collected at each measurement session were then analysed to identify common features in each of the images, in order to register the OCT image locations for each subsequent session, with the OCT image locations in measurement session 1 (middle). The best quality central cross-sectional image from the first measurement session, and the corresponding B-scan in each of the subsequent measurement sessions was then segmented to delineate the anterior conjunctival boundary (ACB, blue line), the posterior scleral boundary (PSB, red line) and the anterior scleral boundary (ASB, green line), in order to calculate the conjunctival thickness (distance from ASB to ACB) and scleral thickness (distance from PSB to ASB), at 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 mm from the scleral spur (bottom).

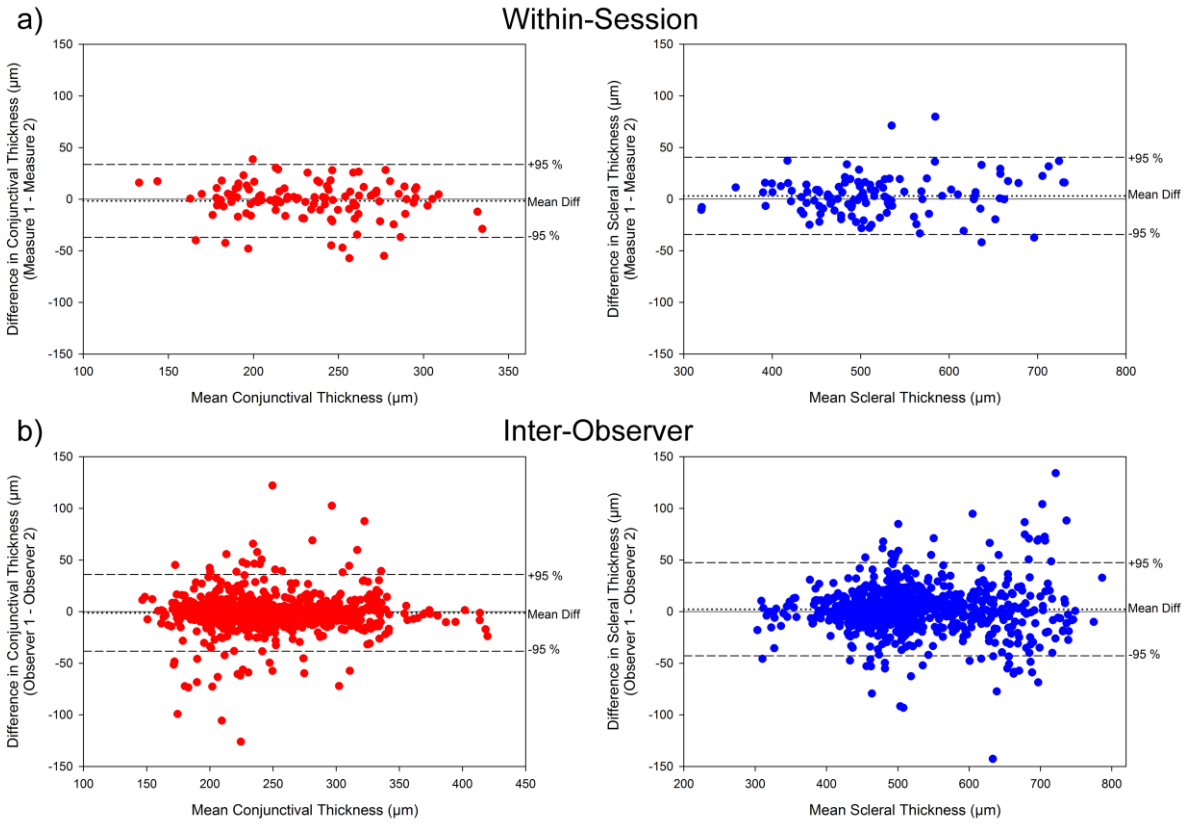

Figure 2: Overview of the within-session (a) and inter-observer (b) repeatability for the measurements of conjunctival thickness (red symbols) and scleral thickness (blue symbols). Bland-Altman[28] plots, demonstrating the mean difference (dotted line) and the 95% limits of agreement (dashed line) of the differences are illustrated.

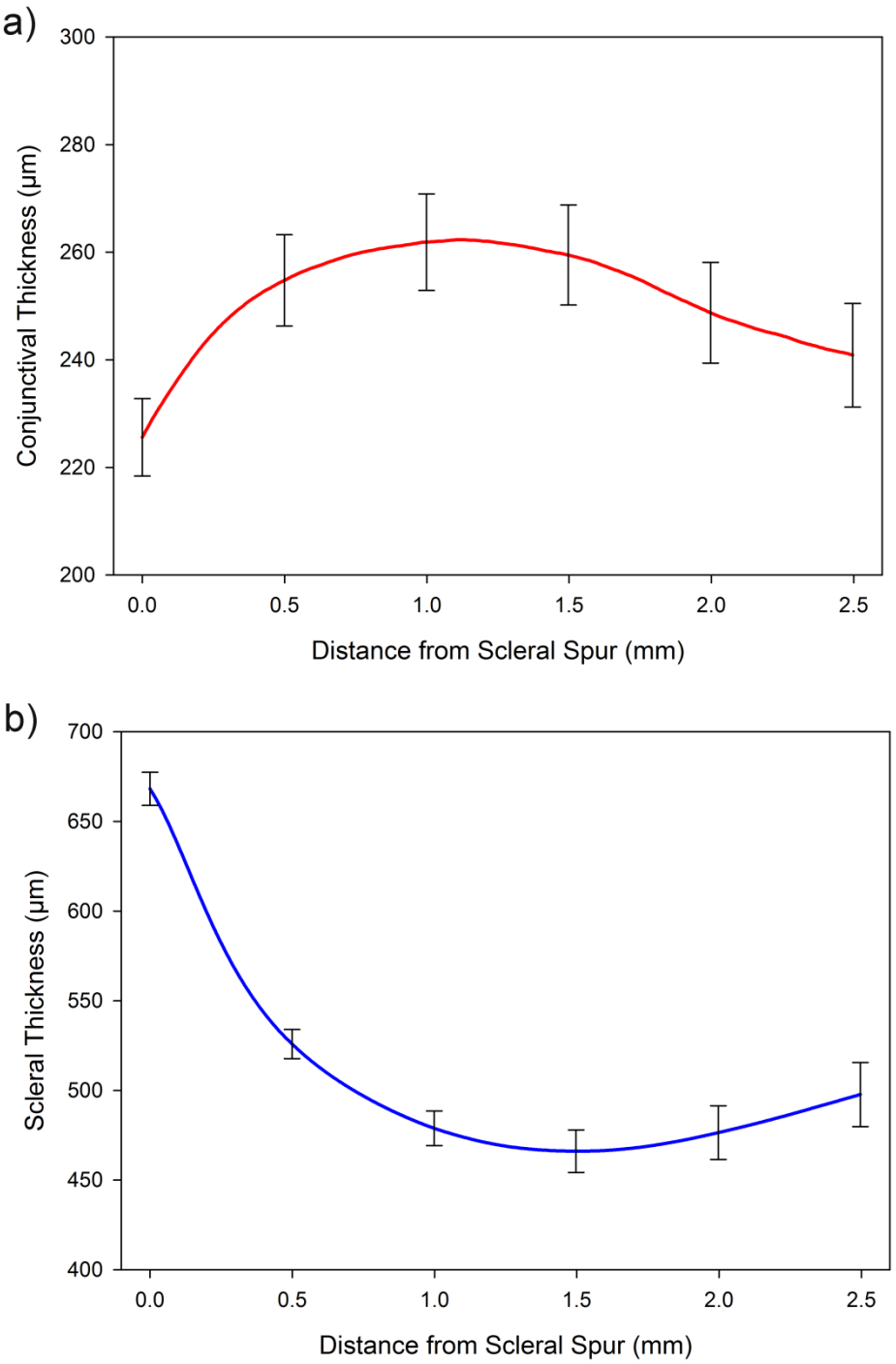

Figure 3: Mean profile of the temporal anterior conjunctival (red line, a) and scleral (blue line, b) thickness with distance from the scleral spur for 19 young adults. The mean thickness profile is derived from the average of all seven measurement sessions for each participant. Error bars represent the standard error of the mean.

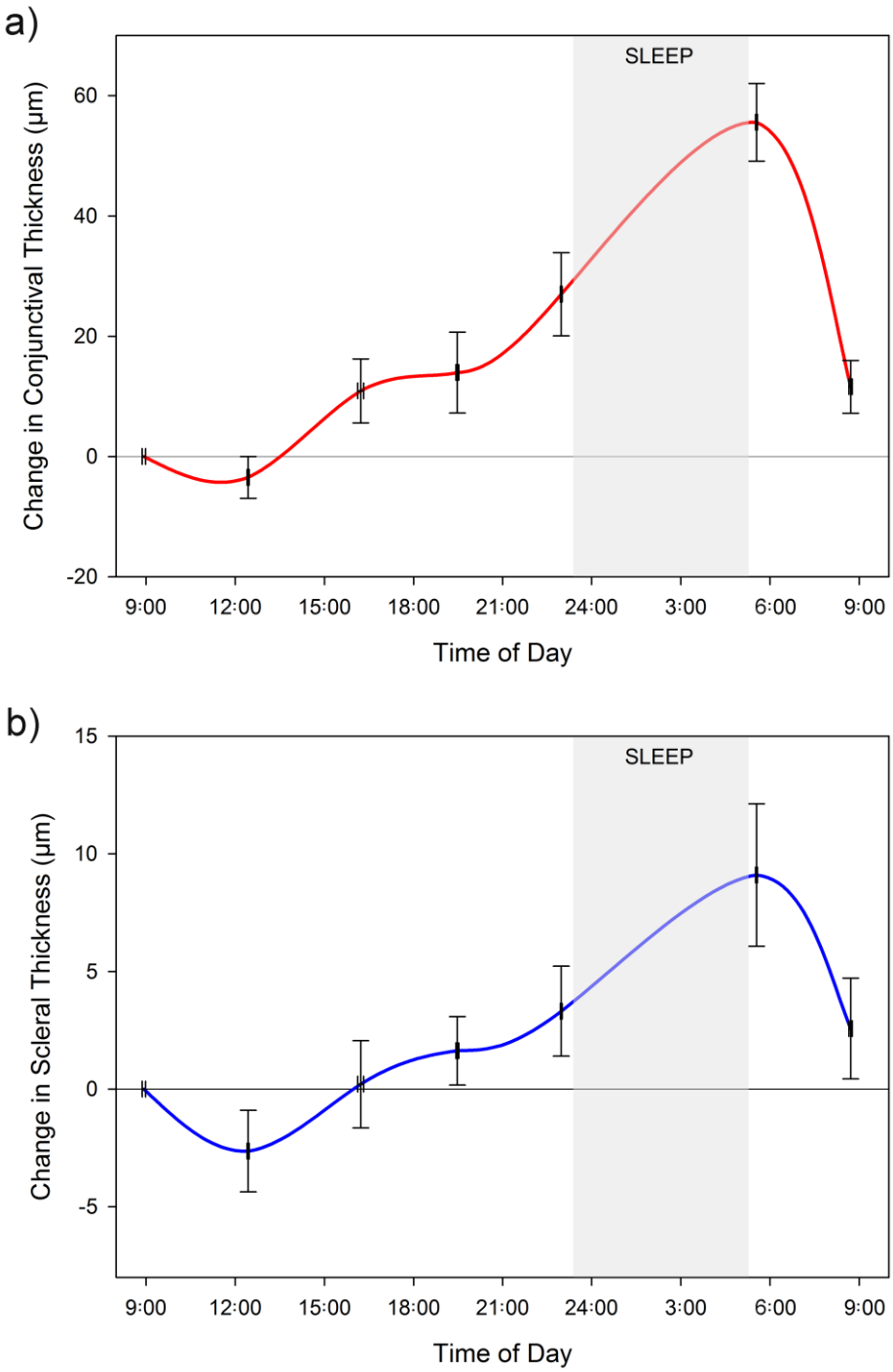

Figure 4: Average change from baseline in the temporal anterior conjunctival (red line, a) and scleral (blue line, b) thickness (averaged across all 6 locations with respect to the scleral spur) over the course of the day for 19 young adults. Error bars represent the standard error of the mean. Note that the Y-axis scale is different in the conjunctival (a) and scleral (b) thickness changes.

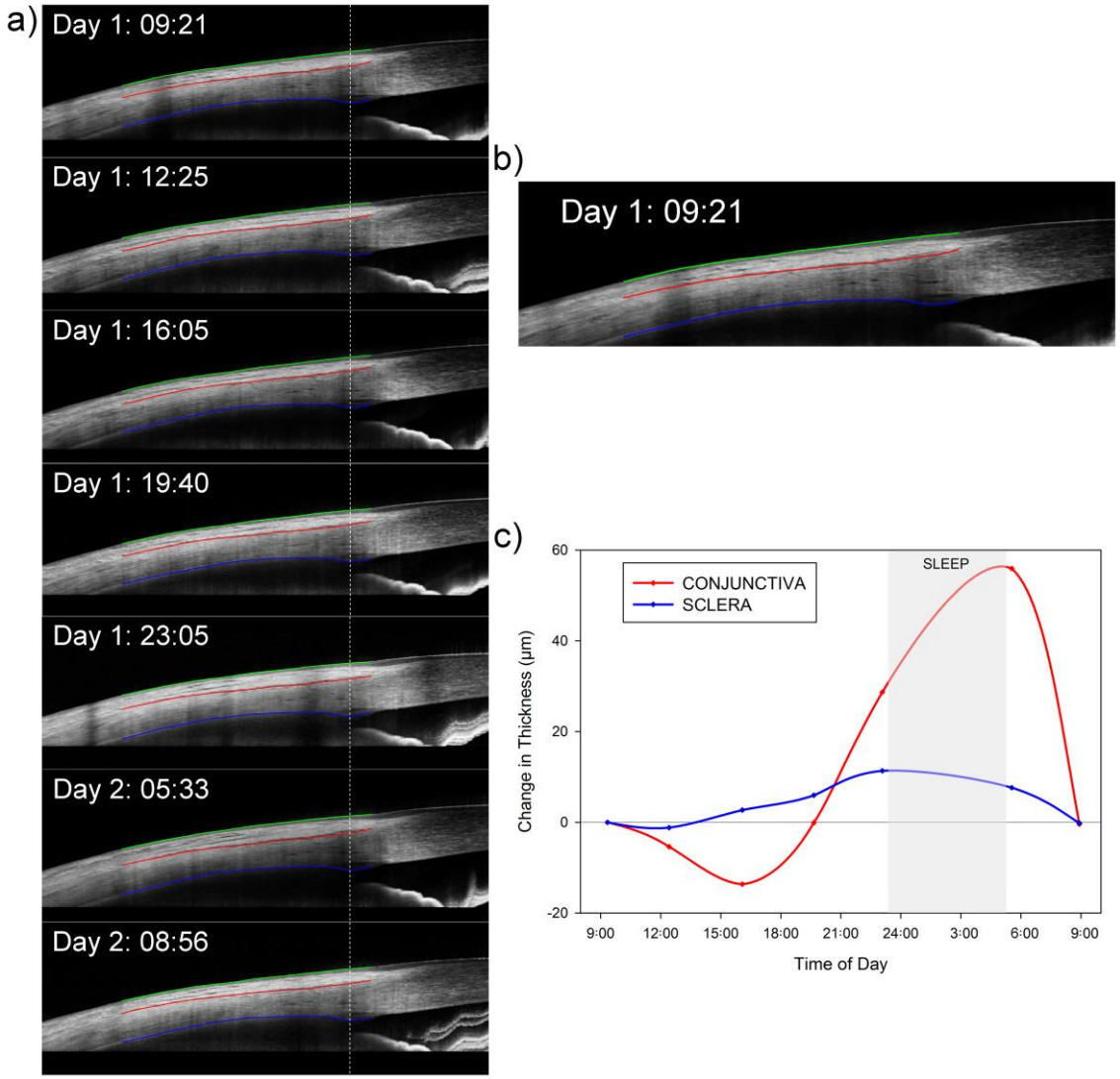

Figure 5: OCT images of the anterior sclera and conjunctiva at each measurement session for a representative subject (a,b) along with the mean changes in thickness from baseline over the 24 hours of measurements for this subject (c). The white dashed lines in (a) indicate the position of the scleral spur. An animation of the OCT images at each session is shown in (b) to highlight the variations occurring in the scleral and conjunctival tissue over the 24 hour period, and can be viewed in the supplemental file (Figure_5_Animated.gif).

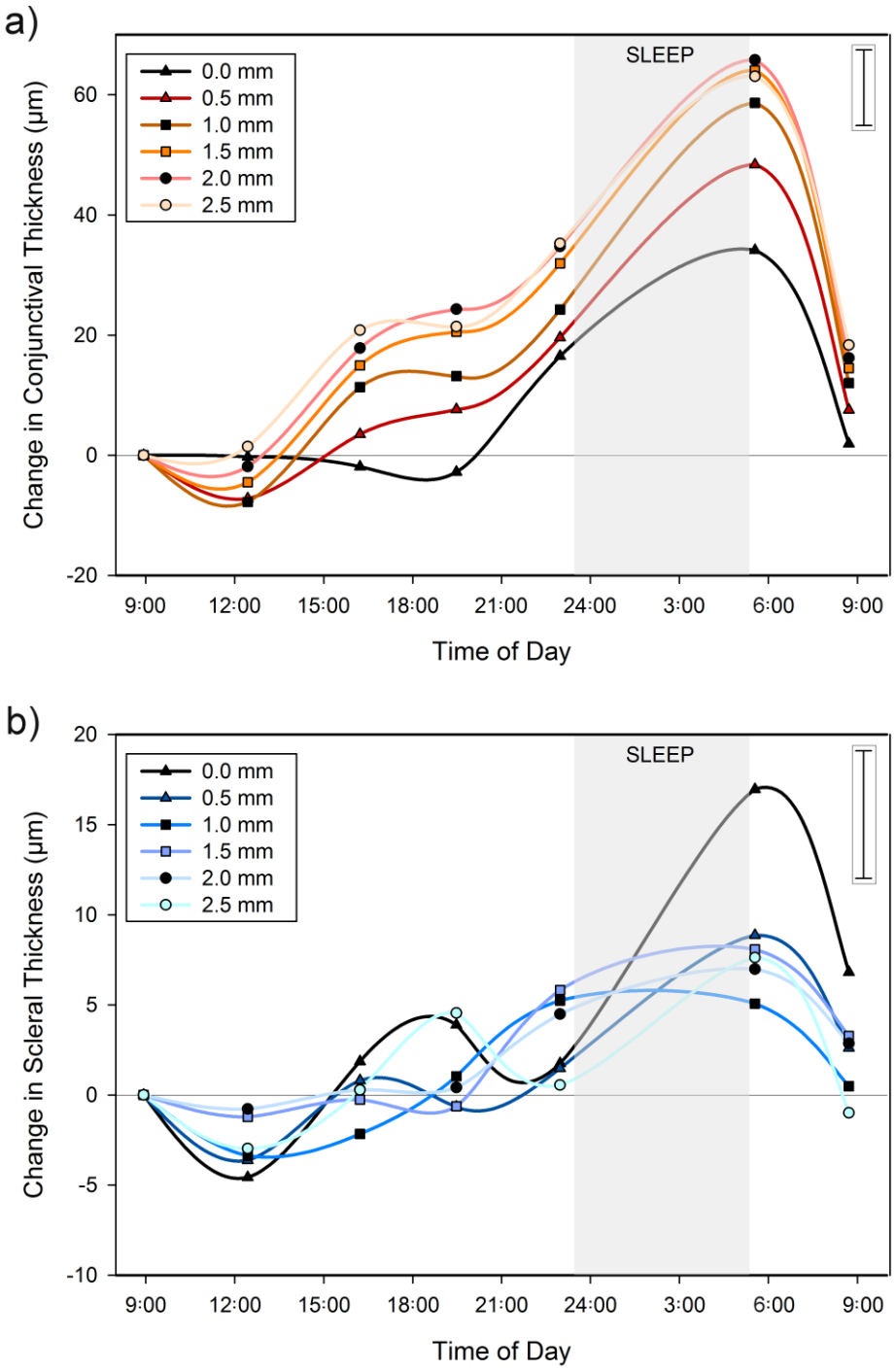

Figure 6: Average change from baseline in the temporal anterior conjunctival (a) and scleral (b) thickness, for each of the 6 measurement locations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mm from the scleral spur) over the course of the day for 19 young adults. Inset in top right hand corner of (a) and (b) illustrates the magnitude of the average standard error of the mean for all measurement locations over all measurement time points**.** Note that the Y-axis scale is different in the conjunctival (a) and scleral (b) thickness changes.

Tables:

Table 1: Overview of the within-session and inter-observer repeatability of the OCT image analysis.

		COV	Mean (SD) difference (µm)	95% limits of agreement (µm)
Within-Session	Conjunctival Thickness	5%	-2 (18)	34 to -37
	Scleral Thickness	3%	+3 (19)	41 to -34
Inter-Observer	Conjunctival Thickness	5%	-1 (19)	36 to -38
	Scleral Thickness	3%	+2 (23)	47 to -43

COV = coefficient of variation

Table 2: Mean (SD) thickness and diurnal amplitude of change in thickness of the temporal anterior conjunctiva and sclera at various locations with respect to the scleral spur.

		Measurement location (distance from scleral spur)						
		0.0 mm	0.5 mm	1.0 mm	1.5 mm	2.0 mm	2.5 mm	Mean of all locations
Conjunctival thickness	Mean (SD) thickness (µm)	226 (31)	255 (37)	262 (39)	259 (41)	249 (41)	241 (42)	249 (42)
	Mean (SD) diurnal amplitude (µm)	56 (17)	66 (26)	78 (32)	85 (35)	84 (36)	81 (36)	69 (29)
Scleral Thickness	Mean (SD) thickness (µm)	668 (40)	526 (36)	479 (42)	466 (52)	476 (65)	498 (78)	519 (46)
	Mean (SD) diurnal amplitude (µm)	52 (21)	33 (11)	29 (10)	28 (9)	26 (11)	28 (12)	21 (8)